The detection of mating type genes of Tuber melanosporum in productive and non productive soils

The detection of mating type genes of *Tuber melanosporum* in productive and non productive soils

Elisa Zampieri[a], Roberta Rizzello[a], Paola Bonfante[a, b], Antonietta Mello[b, ,]

[a] Dipartimento di Biologia Vegetale dell'Università di Torino, Viale Mattioli 25, 10125 Torino, Italy

[b] Istituto per la Protezione delle Piante del CNR, UOS Torino, Viale Mattioli 25, 10125 Torino, Italy

Abstract

Truffles are hypogeous ectomycorrhizal fungi. Of all the different species, *Tuber melanosporum* is one of the most popular on the truffle market. The aim of this work was to set up a protocol in order to check the fertility of a *T. melanosporum* ground. The correlation between its abundance in soil, the presence of mating type genes and productivity was investigated. Soil sampling was conducted in a truffle-ground over two periods of the *T. melanosporum* life cycle, and under two different host species, to verify whether the time and plant species can affect the quantity of mycelium in the soil. An effective quantitative PCR protocol was set up and employed to the investigated truffle-orchard.

We found a statistically significant difference in *T. melanosporum* abundance between the productive and unproductive soils collected in April. Mating type genes for *T. melanosporum* were detected under productive and formally productive trees and generally not under unproductive trees even though *T. melanosporum* was detected. In all the three situations the mating type genes were detected when more than 0.3 ng of *T. melanosporum* DNA was present. Our results suggest combining these approaches to increase knowledge on the fertility of truffle orchards.

Highlights

► We present a protocol in order to check the possibility of a ground to produce *T. melanosporum*. ► Soil samples from a truffle-ground were investigated. ► A quantitative real time PCR was set up to quantify mycelium in the soil. ► The mating type genes were identified when more than 0.3 ng of *T. melanosporum* was present. ► The correlation between *T. melanosporum* abundance and productivity is also discussed.

Keywords

- *Tuber melanosporum*;
- Mating type genes;
- qPCR;
- Truffle-ground plantation;
- Soil

1. Introduction

One of the challenges of studying fungal ecology in soil involves setting up techniques and sampling strategies that can take into account the high complexity and heterogeneity of this environment. Among soil fungal communities, ectomycorrhizal fungi have been investigated in great extent over the last 15 years (Bonfante et al., 2010). Apart studies on fruiting bodies, analyses of the below-ground communities have also been conducted on infected root tips (Horton and Bruns, 2001). However, they have provided very little information on the distribution and diversity of fungal mycelium in soil. For instance, *Tuber magnatum* mycelium resulted to be more widespread than could be inferred from the distribution of the truffles and ectomycorrhizas (Zampieri et al., 2010).

Quantitative real-time PCR (qPCR) has become common practice in the detection and quantification of various organisms, since it is a very powerful, cultivation-independent, rapid and sensitive method (Salvioli et al., 2008 and Taylor et al., 2010). The quantification of the target amount in unknown samples can be calculated by generating a standard curve (Song et al., 2002). Over the past ten years, at least five papers have reported the detection, distribution and abundance of a specific ectomycorrhizal fungus through qPCR in the soil (Guidot et al., 2002, Landeweert et al., 2003, Parladé et al., 2007, Suz et al., 2008 and De la Varga et al., 2012). Suz et al. (2008) attempted to correlate mycelia abundance and other factors related to truffle productivity in *Tuber melanosporum–Quercus ilex* orchards. This black truffle is one of the most popular marketed truffle species because of its taste and aroma. Natural production is almost exclusively in France, Spain and Italy, which are the main distribution areas for *T. melanosporum* (Mello et al., 2006). Nevertheless, as truffle production declined spectacularly in the 20th century and the ecological requirements for this species are relatively well known, cultural practices have been developed in these and other countries throughout the world (Reyna-Domenech and Garcia-Barreda, 2009). Truffle cultivation requires long-term investments: production usually begins 6–10 years after seedling inoculation. The fruiting body (truffle) is usually collected in the burned area, an area devoid of vegetation near or around the host trees, where the truffle competes with other ectomycorrhizal fungi (Napoli et al., 2010). Nevertheless, potential truffle yield remains highly unpredictable. Recently, the sequencing of the *T. melanosporum* genome (Martin et al., 2010) has opened new frontiers, and this has allowed us to identify the genes that are involved in the reproductive process and to demonstrate the heterothallic nature of this fungus (Rubini et al., 2011a and Rubini et al., 2011b). These findings are highly relevant in the truffle cultivation since the retrieval of both mating type genes in a stand will ensure that it is fertile.

The aim of our work was to set up a method to check the fertility of a *T. melanosporum* ground. This could be applied in truffle-culture programs and could assist truffle operators in the management of their orchards. Since truffle cultivation first started (Chevalier, 1994), it has only been possible to check for the introduced *Tuber* species, and analyze the ectomycorrhizal symbionts after seedling inoculation and before the harvest of the fruiting bodies, through morphological and PCR techniques (Mello et al., 2006). Initially, this was only made by searching for the *T. melanosporum* morphotypes, but then the molecular methods used for fruiting body identification became tools that could be used to attest the occurrence of *T. melanosporum* in mycorrhizal roots (Baciarelli-Falini et al., 2006).

In this work, we wanted to discover whether there is a correlation between *T. melanospoum* mycelium abundance, the presence of mating type genes and productivity. Although a protocol for *T. melanospoum* quantification in the soil was already available (Suz et al., 2008), the primers used were not specific for *T. melanosporum*; therefore we set up a new qPCR protocol using *T.*

melanospoum specific primers designed by Bonito (2009). The aims were: (1) to assess the quantity of *T. melanosporum* in the soil of a model truffle-ground; (2) to verify whether the detection of both mating type genes mirrors productivity and depends on the quantity *of T. melanospoum*. In order to answer these questions, soil samples were collected in one truffle-ground. The ground was used as a model since it presented three distinct situations: productive, non-productive and formally productive host trees. We hypothesized that different sites would show different quantities of *T. melanosporum* in the soil, and that the highest quantities would be detected under productive trees. Sampling was made over two periods of the *T. melanosporum* life cycle and under two different host trees (*Quercus pubescens* and *Corylus avellana*) to verify whether these conditions could affect the quantity of *T. melanospoum* in the soil.

2. Materials and methods

2.1. Soil sampling and DNA extraction

The soil was sampled in a *T. melanosporum* plantation, at a depth of 10–15 cm and at 1 m from the tree, and stored at −80 °C. The plantation was established in 1994 and organized in two sub-areas 500 m apart (Fig. 1). Twenty soils were harvested in Moncalvo in a well managed truffle-ground (Asti, Piedmont, 45°3′4,32″N; 8°15′58,68″E) in April 2010, when fructification was over. These soil samples were collected around three oaks (*Quercus pubescens*) and two hazelnuts (*Corylus avellana*) (Table 1). Four collection sites were identified and labeled around each plant. The oak trees, placed in a sub-area, were called 1-A, 2-A and 3-A, while the hazelnut trees, placed in another sub-area 500 m away from the first, were called 4-A and 5-A. Tree 1-A was a productive plant for six years until 2002/2003, while Trees 2-A and 4-A remain productive. Trees 3-A and 5-A have never been productive. The same truffle-ground sub-areas were sampled in October 2010, during the fructification period; 24 soils were harvested around three oaks and three hazelnuts. Four collection sites were identified and labeled around each plant. For each sub-area one plant was sampled again: the trees number 2 and 4. The oak trees were called 2-O, 8-O and 9-O, while the hazelnuts were called 4-O, 11-O and 12-O. Plants 2-O, 8-O, 4-O and 11-O are productive, while the others (9-O and 12-O) are non-productive (Table 1). The five oaks were at a minimum distance of 3 m and at maximum distance of 12 m, while the four hazelnuts were at a minimum distance of 6 m and at a maximum distance of 150 m (Fig. 1). All the plants, except the unproductive ones, became productive four–five years after the plantation. The yield for each productive plant was approximately 300 g.

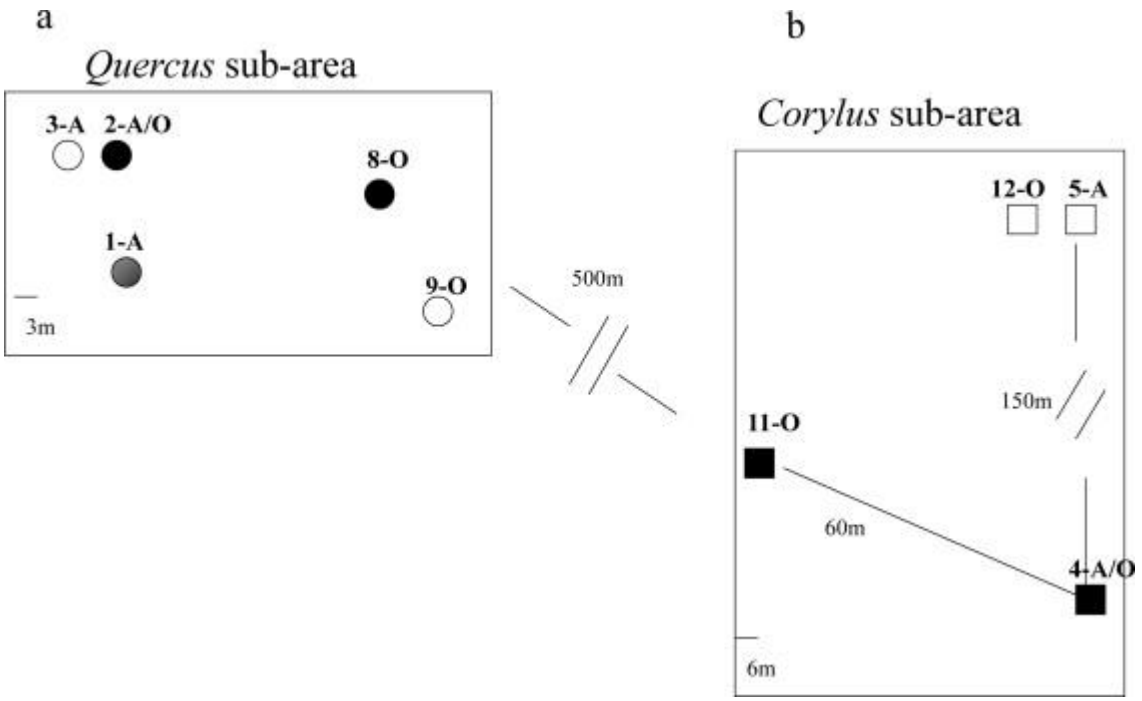

Fig. 1. Map of the two sub-areas in Moncalvo truffle-ground. The panel (a) shows the *Quercus* sub-area, while the panel (b) shows the *Corylus* sub-area. The two sub-areas are 500 m distant. The trees are indicated with a circle (*Q. pubescens*) and a square (*C. avellana*). The productive plants are black forms, the unproductive ones are white forms and the formally productive host tree is in gray.

Table 1. Quantification of *T. melanosporum* DNA in the Moncalvo truffle-ground by *T. melanosporum* specific primers (Bonito, 2009), employed in qPCR. The plant species considered in April are indicated with (A) while those considered in October are indicated with (O). "No detection" means that the soil DNA did not amplify in conventional PCR, while "–" means that the quantification was not possible. The presence (×) of mating type genes (MAT1-1-1 and MAT1-2-1) is shown.

Plant species	Soil samples	*Tuber melanosporum* DNA quantity (ng/µL)	MAT 1-1-1	MAT 1-2-1
Quercus pubescens formally productive 1-A	Soil 1	1.251		×
	Soil 1bis	0.655		×
	Soil 1tris	0.664		×
	Soil 1tetra	0.701		×
Quercus pubescens productive 2-A	Soil 2-A	1.081		×
	Soil 2-A bis	2.085		×
	Soil 2-A tris	0.790		×

Plant species	Soil samples	*Tuber melanosporum* DNA quantity (ng/µL)	MAT 1-1-1	MAT 1-2-1
Quercus pubescens unproductive 3-A	Soil 2-A tetra	1.170		×
	Soil 3	0.061		
	Soil 3bis	0.087		
	Soil 3tris	0.056		
	Soil 3tetra	0.046		
Corylus avellana productive 4-A	Soil 4-A	1.032	×	
	Soil 4-A bis	0.999	×	
	Soil 4-A tris	1.765	×	
	Soil 4-A tetra	0.504	×	
Corylus avellana unproductive 5-A	Soil 5	0.025		
	Soil 5bis	0.030		
	Soil 5tris	0.022		
	Soil 5tetra	0.040		
Quercus pubescens productive 2-O	Soil 2-O	0.365		×
	Soil 2-O bis	0.407		×
	Soil 2-O tris	0.539		×
	Soil 2-O tetra	0.979		×
Quercus pubescens productive 8-O	Soil 8	0.050		×
	Soil 8bis	0.906		×
	Soil 8tris	0.721		×
	Soil 8tetra	0.953		×
Quercus pubescens unproductive 9-O	Soil 9	0.602		×
	Soil 9bis	No detection		
	Soil 9tris	–		

Plant species	Soil samples	*Tuber melanosporum* DNA quantity (ng/µL)	MAT 1-1-1	MAT 1-2-1
Corylus avellana productive 4-O	Soil 9tetra	0.004		
	Soil 4-O	3.520	×	
	Soil 4-O bis	3.075	×	
	Soil 4-O tris	1.105	×	
	Soil 4-O tetra	2.083	×	
Corylus avellana productive 11-O	Soil 11	1.893		×
	Soil 11bis	4.787		×
	Soil 11tris	–		×
	Soil 11tetra	0.324		×
Corylus avellana unproductive 12-O	Soil 12	No detection		
	Soil 12bis	No detection		
	Soil 12tris	No detection		
	Soil 12tetra	No detection		

Soil samples coming from the four collection sites around six plants (2-A, 3-A, 4-A, 5-A, 9-O, 4-O) were mixed to determine soil chemical features. The plants chosen for chemical analysis represent different situations respect to plant species (*Quercus pubescens* and *Corylus avellana*), geographical positions (3 plants for sub-area), resampling period (April and October), productivity and unproductivity. Soil chemical features were measured by Agrochemistry Lab of Piedmont Region and reported in Table 2.

Table 2. Chemical properties of the soils sampled around plants in Moncalvo truffle-ground. The plant species considered in April are indicated with (A) while those considered in October are indicated with (O).

	***Quercus pubescens* 2-A productive**	***Quercus pubescens* 3-A unproductive**	***Corylus avellana* 4-A productive**	***Corylus avellana* 5-A unproductive**	***Quercus pubescens* 9-O unproductive**	***Corylus avellana* 4-O productive**
pH	8	7.9	8.1	7.8	7.9	8.2
Texture	Sandy loam	Sandy loam	Clay loam	Clay loam	Clay loam	Clay loam

	Quercus pubescens 2-A productive	*Quercus pubescens* 3-A unproductive	*Corylus avellana* 4-A productive	*Corylus avellana* 5-A unproductive	*Quercus pubescens* 9-O unproductive	*Corylus avellana* 4-O productive
C/N	10.3	11.1	10.3	11.4	11.4	11.9
Organic carbon %	1.12	1.72	0.95	2.10	2.17	1.30
Total nitrogen %	0.11	0.15	0.09	0.18	0.19	0.11
Total calcium carbonate %	20.7	20.3	24.3	29.5	29.9	23.1
Cation-exchange capacity meq/100 g	14.7	15.6	22.1	19	18.9	22.5
Exchangeable calcium meq/100 g	15.51	15.55	22.43	20.61	20.26	23.68
Exchangeable magnesium meq/100 g	1.00	1.04	1.68	2.12	1.59	1.87
Exchangeable potassium meq/100 g	0.77	0.62	0.38	0.74	0.68	0.40
Available phosphorus p.p.m.	24	22	6	11	20	6

DNA was extracted using the Fast DNA Spin kit for soil (Qbiogene), according to Luis et al. (2004). DNA quality was tested *via* the PCRs, which were performed with the fungal ITS (internal transcribed spacer) universal primers ITS1-F (Gardes and Bruns, 1993) and ITS2 (White et al., 1990). The mix consisted of: 10× buffer (Sigma) (2.5 μL), 2.5 mM dNTPs (2 μL), 10 μM primer f (1 μL), 10 μM primer r (1 μL), water (15.2 μL), Red Taq 1 U μL^{-1} (Sigma–Aldrich, Milan, Italy) (0.7 μL) and DNA (2 μL). The PCR was carried out on a Gene Amp PCR System 2700 (Applied Biosystems, Milan, Italy) thermocycler with denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s, and an extension at 72 °C for 5 min.

2.2. *T. melanosporum* detection

DNAs from all the collected soil samples were tested with two *T. melanosporum* specific primer pairs: T.mel_for and T.mel_rev (Bonito, 2009), used at an annealing temperature of 56 °C; ITSML and ITS4LNG (Paolocci et al., 1999) at an annealing temperature of 58 °C. Four *T. melanosporum*

fruiting bodies, which were harvested in Moncalvo under plants number 2-O and 11, were used in positive control reactions. The same PCR mix employed with the ITS universal primers was used (see above).

2.3. Mating type detection

The soil samples and the four *T. melanosporum* fruiting bodies were analyzed using the mating type gene primers, developed by Rubini et al. (2011a).

2.4. Sequence analysis

All the PCR products were checked on 1–1.2% agarose gel, purified (Qiaquick PCR purification kit, Qiagen) and sequenced (DiNAMYCODE, Turin, Italy). The sequences were edited with the Sequencher program, version 4.1.4 (Gene Codes Corporation) and compared with the GenBank database to verify whether they corresponded to *T. melanosporum*. The ITS sequences for the soils were deposited in GenBank with the following accession numbers: from FR820785 to FR820829.

2.5. Quantitative real-time PCR (qPCR)

Quantitative PCR (qPCR according with Bustin et al., 2009) was carried out with a StepOne apparatus (Applied Biosystem), using the T.mel_for and T.mel_rev primer pair (Bonito, 2009). Each PCR was conducted on a total volume of 20 μL, containing 1 μL of soil DNA (0.47–2.67 ng/μL), 10 μL of SYBR Green Reaction Mix and 2 μL of each primer (3 μM) using a 48-well plate. DNA-free controls were run for each experiment. The following PCR program, which includes the calculation of a Melting curve, was used: 95 °C for 10 min, 45 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, 95 °C for 15 s, 60 for 1 min, and 95 °C for 15 s. All the reactions were performed for three technical replicates (*e.g.*, <5%). Each standard curve was generated using 10-fold serial dilutions of up to 10^{-5} of *T. melanosporum* fruiting body genomic DNA extract (298 ng/μL), putting 1 μL of each serial dilution into 9 μL of a soil DNA (a Moncalvo soil, in which the absence of *T. melanosporum* was verified with different primer pairs), assuming that the amplification efficiency of the standard DNA was equal to the amplification efficiency of the sample target DNA. The formation of a specific amplified product was confirmed by analyzing the melting curve. *T. melanosporum* in the soil samples was converted to *T. melanosporum* ng in 1 μL of soil DNA, on the basis of the standard curve, by comparing the Ct values of the unknown samples with those of the standards.

2.6. Statistical analysis

Standard one-way ANOVA and a LSD (Least Significant Difference) *post hoc* test were used to compare the data. The results were considered significant at $P < 0.05$. All the statistical analyses were performed with Systat 9 (Systat Software Inc.).

3. Results

DNA was successfully extracted from the soil samples considered in the work (Fig. 1). Their amplificability was verified with the fungal ITS universal primers, which gave the expected band on all the 44 soil samples (data not shown).

Presence of *T. melanosporum* was checked in the soil samples and its abundance was quantified. Mating type genes were subsequently searched for in all the soil samples to verify whether the

mating type detection was limited by the quantity of *T. melanosporum* mycelium. The chemical features of the soil under six selected plants were successfully analyzed and compared (Table 2). The features under two plants, placed at a short distance (Fig. 1), a productive one (*Quercus pubescens* 2-A) and an unproductive one (*Quercus pubescens* 3-A), showed very similar values regardless of the productivity and of the *T. melanosporum* DNA quantity found. The chemical features of the soil under the plants at a distance showed slightly different values, regardless of the plant species and the productivity. The soil sampled under *Corylus avellana* 4, in two periods (April and October), showed similar chemical properties.

3.1. *T. melanosporum* detection

Since the primers specifically tested by Bonito (2009) on *T. melanosporum* fruiting bodies seemed to be suitable for use in qPCR (*i.e.*, short length PCR product, no dimer formation), they were tested to verify their specificity also for *T. melanosporum* in soils. They gave the expected signal in 39 out of 44 soils (data not shown). Given that these primers were successful in detecting *T. melanosporum* mycelium, they were then employed to quantify it in all the soil samples.

We wondered whether the soils that did not show a signal with Bonito's primers could still contain *T. melanosporum*. Therefore, we tested these soils with another pair of primers specifically designed for *T. melanosporum* by Paolocci et al. (1999). The Paolocci primers didn't amplify *T. melanosporum* from all the samples either, but through this parallel comparison, we observed that another 4 samples gave the expected band. Sequences from these and the above 39 soils confirmed *T. melanosporum* amplification. *T. melanosporum* was detected in all the soils harvested in Moncalvo, except in 9bis.

3.2. Mating type

In order to identify both mating types, the two pairs of primers developed by Rubini et al. (2011a) were employed. The soil DNAs from Moncalvo were processed as were the four *T. melanosporum* fruiting body DNAs.

The 421 bp band, corresponding to MAT1-1-1, was present in the soils around the plants 4 and 10, while the 550 bp band, corresponding to MAT1-2-1, was present in the soils around plants 1-A, 2-A and -O, 8-O, 11-O, in a single collection site around plant 9-O (Table 1), and in the fruiting bodies from plants 2-O and 11-O. Although different PCR conditions were changed (extension time, annealing temperature), it was not possible to detect the mating type in the non-productive soils of both the April and October samplings, except in a single site collected in October (Table 1).

3.3. *T. melanosporum* quantification

Quantitative real time PCRs were performed in order to quantify the *T. melanosporum* in the soil, using the primers developed by Bonito (2009); the efficiency of each reaction was >98%.

Each Moncalvo soil DNA was processed in qPCR. The quantification results are shown in Table 1 and Fig. 2. The quantity of the *T. melanosporum* DNA in Moncalvo was between 0.022 and 2.08 ng in April 2010 and between 0.004 and 4.78 ng in October 2010 for 1 μL of soil DNA. Quantification was only obtained in two biological replicates in plant 9-O (it was between 0.004 ng and 0.6 ng), but it was not possible to quantify in any of the four replicates around plant 12-O, even though *T. melanosporum* had been detected, using the primers developed by Paolocci et al. (1999).

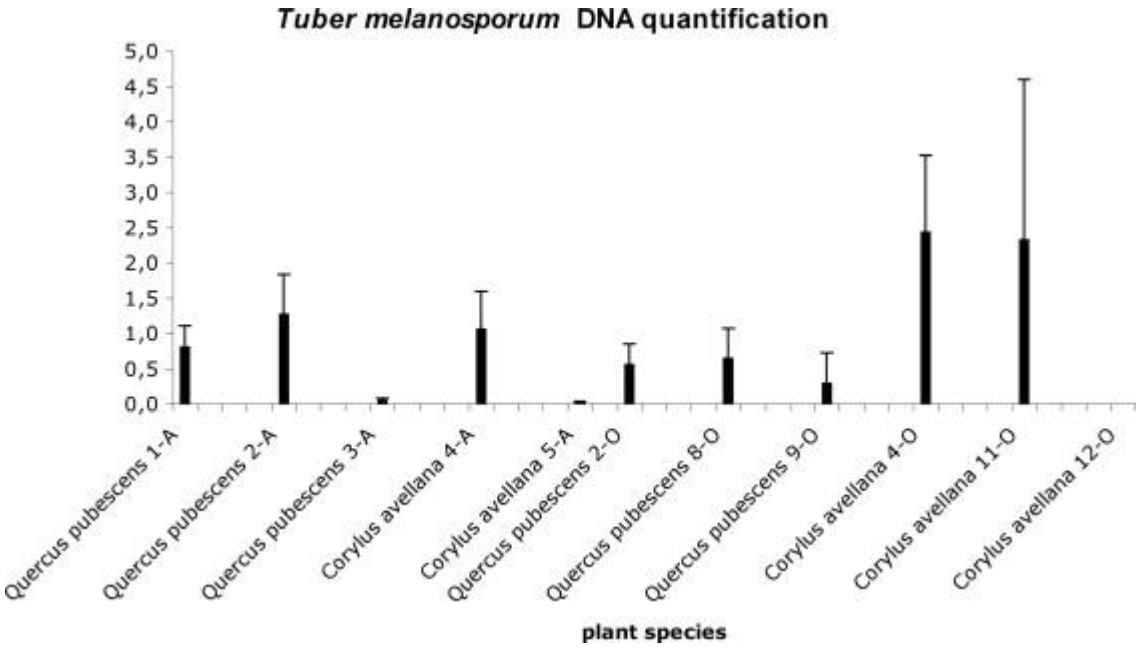

Fig. 2. *T. melanosporum* DNA quantity. *T. melanosporum* DNA quantity in Moncalvo soils in April (A) and in October (O) sampling. On the *x*-axis the plant species, on the *y*-axis the quantity calculated as an average coming from each biological replicate.

The quantification data were processed by statistical analysis. In April, the amount of *T. melanosporum* DNA in productive soils was statistically higher than in non-productive soils, while there was no difference between a productive and a formally productive soil (Tree 2-A *vs* Tree 1-A). Moreover, the quantity of *T. melanosporum* was not statistically different between the soils from the hazelnuts and oaks. In fact, the quantities around plants 3-A and 5-A were not statistically significant different ($P > 0.05$) and neither were those around plants 1-A, 2-A and 4-A ($P > 0.05$). In the statistical analysis of October, the non-productive soils could not be used, because quantification was only possible around unproductive Tree 9-O for two replicates and the *T. melanosporum* amount was not enough to be quantified around unproductive Tree 12-O. Considering only the data from the productive trees, there was a statistically significant difference between the DNA quantity around the two oaks and that around hazelnut number 4-O. Since one oak (no. 2) and one hazelnut (no. 4) were sampled twice, in April and in October, we compared the *T. melanosporum* DNA quantity between 2-A and 2-O and between 4-A and 4-O. There was no statistically significant difference ($P > 0.5$) between the two seasons for both oak and hazelnut.

4. Discussion

A combination of various approaches has allowed us to define the limits of a protocol that can be applied to truffle-culture, and to help/inform truffle operators about the state of their orchards after long-term investments, before the arrival of *T. melanosporum* fruiting bodies. Since production usually begins 6–10 years after the seedlings have been inoculated, management practices are important issues to help to ensure successful black truffle production. Although good practices for cultivation have been established (Reyna-Domenech and Garcia-Barreda, 2009), the heterogeneity of yields is still problematic. In the absence of a protocol, which can offer information on the fertility/sterility of orchards, potential yield remains unpredictable. In a recent study, aimed at deepening our understanding of the vegetative and sexual propagation modes of *T. melanosporum*, Rubini et al. (2011b) monitored the spatial distribution of strains with opposite mating types in a natural plantation, showing a biased distribution and they hypothesized whether this was a factor

that could limit truffle fructification. In spite of the great relevance of this study, no information was given on the relationship among quantity of the *T. melanosporum* mycelium and detection of its mating types in productive, formally productive and non-productive sites.

In this work, we have taken advantage of having access to an orchard with the three aforementioned situations and which could therefore be considered as a model to test the hypothesis that different sites show different amounts of *T. melanosporum* and the presence/absence of its mating types. Soil chemical features around six selected plants were also evaluated to verify whether soil characteristic data mirror molecular data.

The first result of our investigation was that a single primer set may not be sufficient to search for *T. melanosporum* in a stand. The same consideration, therefore, must be done for *T. melanosporum* quantification. The DNA concentration in the soil depends on the distinction between productive and non-productive sites. Mating type genes were not detected under unproductive trees (except in a single site around one plant) even in instances where *T. melanosporum* was detected, whereas they were detected under productive and formally productive host trees. In all the three situations the mating type genes were identified when more than 0.3 ng of *T. melanosporum* DNA was present. Lastly, soil measured chemical features confirm the common requirements of *T. melanosporum* and do not mirror molecular data (productivity and *T. melanosporum* DNA quantity).

4.1. *T. melanosporum* detection depends on the employed primer pair

In this work, we employed two pairs of specific primers for *T. melanosporum*. Although the primers developed by Paolocci et al. (1999) had already been tested on soil DNAs, those developed by Bonito (2009) had not been tested on this type of sample. We have demonstrated that these latter primers are able to detect *T. melanosporum* in soil samples in a specific way. In fact, we identified the fungus in 39 out of 44 soil DNA samples. As, in our experience, four samples, which did not show *T. melanosporum*, amplified with the Paolocci's primers, we suggest using several pairs of primers in programs aimed at checking the presence of the fungus, because their amplification capacity is not the same.

According to Suz et al. (2008), the black truffle mycelium is generally widespread in truffle-grounds, and it also colonizes non-productive sites. We, in fact, found it in 43 out of 44 soils, considering the two possible pairs of primers.

4.2. Mating type detection does not mirror the absence of *T. melanosporum*

The search for mating type genes allowed us to have initial information on the fertility of an area. The two idiomorphs were both found in the Moncalvo truffle-ground, indicating that this site is fertile. One mating (MAT1-2-1) was more frequent in the area, and soil samples of trees close to each other showed the same mating type (*e.g.*, plants 1-A and 2), in agreement with Rubini et al. (2011b), who found that strains with different mating types are not equally distributed in truffle-grounds. The mating (MAT1-1-1) was found only in the hazelnut sub-area under plant 4 in both samplings. Only this sub-area showed the co-presence of the two idiomorphs, which were detected at distance of 60 m. We could not detect the mating type under unproductive trees where the *T. melanosporum* quantity was very low (under 0.3 ng), even though *T. melanosporum* mycelium was detected and several PCR conditions were carried out to improve the mating type gene amplification. This result could be explained by the fact that the amplification of a single locus gene (*i.e.*, MAT) is more difficult than that of a multi locus gene (*i.e.*, ITS) in soil samples containing small quantities of fungus. A mating type gene was identified under an unproductive tree only when the *T. melanosporum* quantification was above 0.3 ng of DNA.

4.3. *T. melanosporum* quantification in relation to plant host and season

We set up a method to employ Bonito's primers (2009) in qPCR, and this allowed us to quantify and to monitor the fungus in a model truffle-ground. The quantification of *T. melanosporum* DNA in soil was likely to have primarily originated from mycelium, although DNA contributions from spores cannot be excluded. Considering the plants analyzed in Moncalvo in April 2010 (three oaks and two hazelnuts), the *T. melanosporum* DNA concentration in the soil was different between the productive and non-productive sites, but not between the still productive and formally productive soils ($P > 0.05$) or between the oaks and hazelnuts ($P > 0.05$). It was not possible to statistically analyze the data from the productive and non-productive soils in October, since we did not obtain sufficient data for the unproductive areas. Because of this, it is only possible to hypothesize a relationship between the quantity of *T. melanosporum* in soil samples and productivity. Considering only the productive sites around the different trees (hazelnuts and oaks), we found statistically significant differences between the two oaks and hazelnut number 4-O ($P < 0.05$), while there was no statistically significant difference between the same oaks and hazelnut number 11-O. This second finding could be explained by the very high variability we found inside the biological replicates of the hazelnut sampling. On the basis of our results in the two seasons, it is possible to hypothesize that the quantity of *T. melanosporum* was not related to the plant species in April, when fructification was over, but was instead related to the plant species in October, during the period of fructification. The root apparatus of *C. avellana* is less deep but wider and forming a broader network than that of *Q. pubescens*, facilitating the contact with *T. melanosporum* mycelium. From this, the priming of truffle life cycle starts with the production of new mycelium, which increases *T. melanosporum* DNA quantity in the soil under hazelnut. One hypothesis to explain the statistically significant difference between oaks and hazelnut could be related to the fact that in October *T. melanosporum* produces its fruiting bodies whose spores concur, even if in less part, to the *T. melanosporum* amount in the soil.

A plant of each species was sampled twice, in April and in October, in order to understand whether the quantity of *T. melanosporum* was affected by the season. The statistical analysis highlighted that the two re-sampled trees did not show any significant difference, and this would seem to suggest that quantity is not related to the season when the same plant species is considered. It would be necessary to analyze the same plants in other months and increase the number of re-sampled plants in the different investigations to confirm this hypothesis. Nevertheless digging the soil of a truffle-ground is not trouble-free, given that *T. melanosporum* is an expensive delicacy and the ground's owner is reluctant to disturb the truffle habitat and risk income loss.

An important result of our investigation is that it was not possible to relate the presence of mating types to the presence of *T. melanosporum*, because the detection of a single locus gene (*i.e.*, MAT) is more difficult than that of a multi locus gene (*i.e.*, ITS). In order to correlate these two factors, it would be necessary to employ the mating type genes in qPCR experiments.

The acceptable values in qPCR are defined by the standard curve; some soil samples showed a faint signal (*e.g.*, soil 11tris) in PCR, which was not confirmed in qPCR. A possible explanation could be connected to the detection limit: the fungus could still be present in the soil, but under the limit established by the standard curve.

In our experience, the mating type genes were always detected with a good signal in the soil when more than 0.3 ng of *T. melanosporum* DNA was quantified.

This work, although preliminary, highlights for the first time the limits of a well-defined protocol in order to check the fertility of a *T. melanosporum* ground. *T. melanosporum* detection and

quantification depend on the primer pair employed; mating type detection is possible above a threshold value of *T. melanosporum* DNA and its missed detection does not necessarily mean that *T. melanosporum* is absent in the soil.

In order to obtain a detailed knowledge on the fertility of a truffle-ground we suggest combining all these approaches, because each of them adds a piece of information to the complex scenario, which characterizes a truffle-ground. So far soil ecological markers of the fertility of a truffle-ground have not been recognized. It is known that *T. melanosporum* grows in calcareous soils with a C/N ratio close to 10 (García-Monteroa et al., 2009). In the absence of other indications, up to now the establishment of a *T. melanosporum* plantation has been exclusively based on soil features. Nowadays molecular analyses can help truffle operators in the management of their plantation by attesting the occurrence of *T. melanosporum* in the truffle-ground, before the harvest of the fruiting bodies. Trained companies, such as spin-off of Universities, can offer PCR service to truffle operators who are not familiar with molecular analyses. However, many abiotic (soil composition, rain, light exposure, temperature, moisture, *etc.*) and biotic factors (fungi, yeasts, bacteria, archea, mesofauna, plants) contribute to the production of truffles (Ceruti et al., 2003). Soil-borne communities living in the rhizosphere may have an impact, facilitating or inhibiting truffle fruiting body production (Mello et al., 2010). The complexity of factors is probably the cause for the heterogeneity of fruiting body yields in truffle-grounds (Kues and Martin, 2011). To have a more complete overview we should focus in the future on the relationships between bacterial and fungal communities associated with *Tuber melanosporum*-productive niches and *T. melanosporum* quantity in the soil.

In conclusion, this study provides a glimpse into the relation between the distribution of *T. melanosporum* mating types and the quantity of the fungus over two periods of the life cycle and under two different host trees in plantation.

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References

Baciarelli-Falini et al., 2006 L. Baciarelli-Falini, A. Rubini, C. Riccioni, F. Paolocci Morphological and molecular analyses of ectomycorrhizal diversity in a man-made *T. melanosporum* plantation: description of novel truffle-like morphotypes Mycorrhiza, 16 (2006), pp. 475–

Bonfante et al., 2010 P. Bonfante, K. Visick, M. Ohkuma Symbiosis in the omics era Environ. Microbiol. Rep., 2 (2010), pp. 475–478

Bonito, 2009 G. Bonito Fast DNA-based identification of the black truffle *Tuber melanosporum* with direct PCR and species-specific primers FEMS Microbiol. Lett., 301 (2009), pp. 171–175

Bustin et al., 2009 S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments Clin. Chem., 55 (2009), pp. 611–622

Ceruti et al., 2003 A. Ceruti, A. Fontana, C. Nosenzo Le specie europee del genere Tuber, Una revisione storica Regione Piemonte, Torino (2003)

Chevalier, 1994 G. Chevalier Evolution des recherches sur les plants mycorhizés par la truffe et perspectives de développement Giorn. Bot. Ital., 128 (1994), pp. 7–18

H. De la Varga, B. Agueda, F. Martínez-Peña, J. Parladé, J. Pera Quantification of extraradical soil mycelium and ectomycorrhizas of *Boletus edulis* in a Scots pine forest with variable sporocarp productivity Mycorrhiza, 22 (2012), pp. 59–68 http://dx.doi.org/10.1007/s00572-011-0382-2

García-Monteroa et al., 2009 L.G. García-Monteroa, A. Quintanab, I. Valverde-Asenjoc, P. Díazd Calcareous amendments in truffle culture: a soil nutrition hypothesis Soil Biol. Biochem., 41 (2009), pp. 1227–1232

Gardes and Bruns, 1993 M. Gardes, T.D. Bruns ITS primers with enhanced specificity for Basidiomycetes: application to identification of mycorrhizae and rusts Mol. Ecol., 2 (1993), pp. 113–118

Guidot et al., 2002 A. Guidot, J.C. Debaud, R. Marmeisse Spatial distribution of the below-ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples FEMS Microbiol. Ecol., 42 (2002), pp. 477–486

Horton and Bruns, 2001 T.R. Horton, T.D. Bruns The molecular revolution in ectomycorrhizal ecology: peeking into the black-box Mol. Ecol., 10 (2001), pp. 1855–1871

Kues and Martin, 2011 U. Kues, F. Martin On the road to understanding truffles in the underground Fungal Genet. Biol., 48 (2011), pp. 555–560

Landeweert et al., 2003 R. Landeweert, C. Veenman, T.W. Kuyper, H. Fritze, K. Wernars, E. Smit Quantification of ectomycorrhizal mycelium in soil by real-time PCR compared to conventional quantification techniques FEMS Microbiol. Ecol., 45 (2003), pp. 283–292

Luis et al., 2004 P. Luis, G. Walther, H. Kellner, F. Martin, F. Buscot Diversity of laccase genes from basidiomycetes in a forest soil Soil Biol. Biochem., 36 (2004), pp. 1025–1036

Martin et al., 2010 F. Martin, *et al.* The black truffle genome uncovers evolutionary origins and mechanisms of symbiosis Nature, 464 (2010), pp. 1033–1038

Mello et al., 2006 A. Mello, C. Murat, P. Bonfante Truffles: much more than a prized and local fungal delicacy FEMS Microbiol. Lett., 260 (2006), pp. 1–8

Mello et al., 2010 A. Mello, L. Miozzi, A. Vizzini, C. Napoli, G. Kowalchuk, P. Bonfante Bacterial and fungal communities associated to *Tuber magnatum*-productive niches Plant Biosyst., 144 (2010), pp. 323–332

Napoli et al., 2010 C. Napoli, A. Mello, A. Borra, A. Vizzini, P. Sourzat, P. Bonfante Tuber melanosporum, when dominant, affects fungal dynamics in truffle-grounds New Phytol., 185 (2010), pp. 237–247

Paolocci et al., 1999 F. Paolocci, A. Rubini, A. Granetti, S. Arcioni Rapid molecular approach for a reliable identification of *Tuber* spp. ectomycorrhizae FEMS Microbiol. Ecol., 28 (1999), pp. 23–30

Parladé et al., 2007 J. Parladé, S. Hortal, L. Pera, L. Galipienso Quantitative detection of *Lactarius deliciosus* extraradical soil mycelium by real-time PCR and its application in the study of fungal persistence and interspecific competition J. Biotechnol., 128 (2007), pp. 14–23

Reyna-Domenech and Garcia-Barreda, 2009 S. Reyna-Domenech, S. Garcia-Barreda European black truffle: its potential role in agroforestry development in the marginal lands of mediterranean calcareous mountains Agroforestry in Europe Current Status and Future Prospects (2009), pp. 295–317

Rubini et al., 2011a A. Rubini, B. Belfiori, C. Riccioni, E. Tisserant, S. Arcioni, F. Martin, F. Paolocci Isolation and characterization of MAT genes in the symbiotic ascomycete *Tuber melanosporum* New Phytol., 189 (2011), pp. 710–722

Rubini et al., 2011b A. Rubini, B. Belfiori, C. Riccioni, S. Arcioni, F. Martin, F. Paolocci Tuber melanosporum: mating type distribution in a natural plantation and dynamics of strains of different mating types on the roots of nursery-inoculated host plants New Phytol., 189 (2011), pp. 723–735

Salvioli et al., 2008 A. Salvioli, E. Lumini, I.A. Anca, V. Bianciotto, P. Bonfante Simultaneous detection and quantification of the unculturable microbe *Candidatus Glomeribacter gigasporarum* inside its fungal host *Gigaspora margarita* New Phytol., 180 (2008), pp. 248–257

Song et al., 2002 P. Song, C.Q. Cai, M. Skokut, B.D. Kosegi, J.F. Petolino Quantitative real-time PCR as a screening tool for estimating transgene copy number in WHISKERS-derived transgenic maize Plant Cell Rep., 20 (2002), pp. 948–954

Suz et al., 2008 L.M. Suz, M.P. Martin, D. Oliach, R.C. Fischer, C. Colinas Mycelial abundance and other factors related to truffle productivity in *Tuber melanosporum–Quercus ilex* orchards FEMS Microbiol. Lett., 285 (2008), pp. 72–78

Taylor et al., 2010 J.M. Taylor, L.J. Paterson, N.D. Havis A quantitative real-time PCR assay for the detection of *Ramularia collo-cygni* from barley (*Hordeum vulgare*) Lett. Appl. Microbiol., 50 (2010), pp. 493–499

White et al., 1990 T.J. White, T. Bruns, S. Lee, J. Taylor Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics M.A. Innis, D.H. Gelfand, J.J. Sninsky (Eds.), PCR Protocols. A Guide to Methods and Applications, Academic Press, San Diego (1990), pp. 315–322

Zampieri et al., 2010 E. Zampieri, C. Murat, M. Cagnasso, P. Bonfante, A. Mello Soil analysis reveals the presence of an extended mycelial network in a *Tuber magnatum* truffle-ground FEMS Microbiol. Ecol., 71 (2010), pp. 43–49